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Node Negative Breast Carcinoma: Hyperprolactinemia and/or Overexpression of p53 as an Independent Predictor of Poor Prognosis Compared to Newer and Established Prognosticators

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The purpose of this study was to investigate a prognostic indicator that can differentiate node negative breast cancer patients (N = 39, T2N0M0) with high risk and low risk for the development of recurrence or metastases. Preoperative plasma prolactin (PRL) was estimated by radioimmunoassay. The expression of PRL, p53, nm23, and c-erbB2 was investigated by immunohistochemical (IHC) localization; cathepsin D (CD, Enzyme Linked Sorbant Assay) and estrogen- and progesterone-receptors (ER and PR, Dextran coated charcoal method) were estimated in the tumor cytosols. The follow-up period was 2–6 years. Statistical comparisons were made between each marker for relapse-free survival (RFS) and overall survival (OS). Of the 39 patients, 18 had hyperprolactinemia (PRL > 20.0 ng/ml plasma), whereas overexpression of p53 was observed in 55% (17/31) tumors. These were independently and in combination associated with a reduced RFS and OS. The rest of the investigated markers did not show promising results. Hyperprolactinemia and/or overexpression of p53 were associated with aggressiveness of the tumor, early disease relapse or metastases, and poor OS in patients with node negative breast cancer. These two markers may enhance our ability to identify node negative breast cancer patients with aggressive tumors, for whom the use of adjuvant chemo and/or endocrine therapy is unequivocally justified.

KEY WORDS: **plasma prolactin, p53, nm23, c-erbB2, cathepsin D, estrogen receptor, progesterone receptor**

INTRODUCTION

Axillary node negative disease presents one of the greatest challenges to oncologists. McGuire et al. [1] demonstrated the importance of studying multiple factors on a single tumor and multifactorial statistical analysis of a large patient database to provide a "framework" from which important biologic and prognostic factors could be used to make treatment decisions. Breast cancer in western Indian females (Gujarat) ranks second as a cancer cause (age standardized rate 22/100,000 population for the year 1991), 70% of which are in advanced stages, i.e., stages III and IV, at the time of diagnosis. In patients with advanced breast cancer, we have recently reported hyperprolactinemia as an indicator of unfavorable prog-

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nosis and plasma prolactin (PRL) as a better marker for monitoring disease activity when compared with a battery of tumor markers [2–4]. However, axillary node negativity (T2N0M0) is observed only in 18–20% of patients with breast cancer and the 5-year relapse-free survival (RFS) is 57%, which is significantly low compared with Western countries [5–7]. This indicates that our set of node negative patients may be a high risk group when compared with the node negative patients of Western countries. Hence, our goal was to determine the clinical utility of both established and newer predictive indicators to identify those patients destined for early disease recurrence and/or death, where the use of adjuvant therapy is unequivocally justified [8]. To our knowledge, this is the first study from western India (Gujarat) investigating the prognostic value of plasma PRL; immunohistochemical (IHC) localization of PRL, p53, nm23, and c-erbB2; cytosolic cathepsin D (CD) and steroid receptors in patients with node negative breast carcinoma.

MATERIALS AND METHODS

Patients

Data for this study were collected from 39 consecutive, unselected, node negative (T2N0M0) breast cancer patients who had undergone breast cancer surgery at The Gujarat Cancer & Research Institute (Ahmedabad) from May 1984 to December 1989. Criteria for consideration in the study were the presence of a primary invasive breast tumor (stage T2), no metastasis in axillary lymph nodes (N0), with at least axillary levels I and II cleared, no distant metastases (M0), unilateral breast cancer, and no other previous or concomitant primary cancer. The patients were staged according to the International Union Against Cancer TNM classification [9]. The distribution of clinical and pathologic data for the entire patient population is listed in Table I.

IHC localization of PRL, p53, nm23, and c-erbB2 were performed in a blinded fashion without knowledge of follow-up clinical issues. The number of patients for whom paraffin-embedded tissue blocks were available and the prognostic indicators studied were: p53 and PRL localized in 31 patients, nm23 and c-erbB2 in 26 patients, and cytosolic CD in 31 patients. Plasma PRL and cytosolic estrogen- and progesterone-receptors (ER and PR) were estimated in 39 patients. The treatment was decided by the clinicians of our institute.

Patient Follow-Up and Methods

The median follow-up duration of the patients was >5 years (range: 2–6 years). Routine examination of all the patients was performed every 3 months for the first 2 years and at 6 monthly intervals thereafter. RFS and overall survival (OS) rates were calculated as the period from surgery until the date of the first disease relapse or death, respectively. RFS was defined as the first documented evidence of new disease manifestation(s) near the area of the original tumor or at distant site(s).

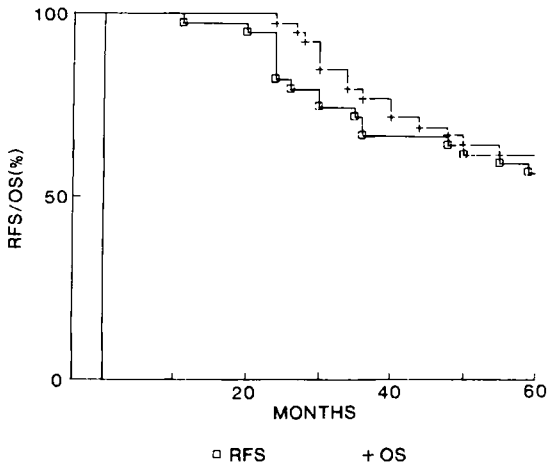

Fig. 1. The relapse-free survival (RFS) and overall survival (OS) of patients with node negative breast cancer.

TABLE I. Clinicopathologic Characteristics of Patients With Node Negative Breast Carcinoma

Age (range 30–80 years)	Median 45 years
Premenopausal	19
Postmenopausal	20
T2N0M0	39
Histological grading (N = 39)	
Grade I	3
Grade II	26
Grade III	10
Treatment	
Surgery	39 followed by
Radiotherapy	2
Tamoxifen	15
Chemotherapy	4
Chemotherapy + Tamoxifen	9
Recurrence or metastatic site (N = 17)	
Local	15
Bone	1
Liver	1

Preoperative blood samples were collected in EDTA-coated tubes (1–2 mg/ml), strictly between 9:00 AM and 12:00 noon to avoid diurnal variation. Blood was separated within 2 hours and plasma was aliquoted and preserved at −70°C until analysis. PRL was estimated with radioimmunoassay kits supplied by Binax Products Co. (Portland, ME). The kit used WHO/RP 75/504 as standard. Results were expressed in ng/ml plasma. The cutoff level for plasma PRL was 20.0 ng/ml; PRL > 20.0 ng/ml was considered as hyperprolactinemic [10]. All estimations were performed in duplicate with intra- and interassay variations of 3–8%.

TABLE II. Distribution of Markers in Patients With Node Negative Breast Carcinoma According to Disease Status

	Patients who developed recurrence or metastasis within 5 years		Patients with NED[a] at the end of 5 years	
	N	(%)	N	(%)
Prolactin > 20.0 ng/ml	13	76.50	5	22.72
p53 positive	10	83.33	7	36.84
Prolactin positive	10	83.33	11	57.89
nm23 negative	5	62.50	5	28.77
c-erbB2 positive	3	30.00	7	43.75
Cathepsin D > 25.62[b]	6	50.00	8	42.10
Estrogen receptor negative	11	64.70	10	45.45
Progesterone receptor negative	8	47.05	6	27.27

[a]No evidence of disease.
[b]pmol/mg protein.
N = number of patients.

Tumor Collection and Histology

The tumor tissue for steroid receptors and CD was selected by an experienced pathologist and its counterpart was fixed for routine histology. Tumor was snap frozen in liquid nitrogen and subsequently stored at −70°C.

ER and PR Assays

ER and PR were measured in the tumor cytosols by the dextran coated charcoal method described elsewhere [11]. Levels >10.0 fmol/mg cytosol protein were taken as positive.

CD Assay

Cytosols left after hormone receptor assays were frozen for a period of 3–6 months at −70°C and thawed once for CD estimation. The protein concentration in the cytosol was estimated by the method of Lowry et al. [12]. After suitable dilution of the cytosols (protein 2–4 mg/ml), CD was estimated by IRMA kit (ELSA-Cath-D) manufactured by CIS Bio International (Gif-sur-Yvette, France). The kit measured total CD (52k, 48k, and 34k proteins), and the levels were expressed as pmol/mg cytosol protein. For CD, median value of 25.62 pmol/mg cytosol protein was used as the cutoff level.

IHC Localization of PRL, p53, nm23, and c-erbB2

All four proteins were determined immunohistochemically on 3–5 μm thick sections of routinely formalin-fixed, paraffin-embedded tissue blocks. Sections were deparaffinized and treated with 3% H_2O_2 to block the endogenous peroxidase activity. The sections were saturated for free nonspecific protein binding sites with normal swine serum for polyclonal antibodies and normal rabbit serum for monoclonal antibodies diluted 1:10 in Tris-buffered saline (TBS, 0.05M Tris-HCl in isotonic solution, pH 7.6) for 20 minutes at room temperature.

For immunostaining of PRL, c-erbB2, and nm23, respective polyclonal rabbit antihuman antibodies were diluted and incubated as follows: PRL (1:50, A 569, DAKO, Carpinteria, CA) for 16 hours at 4°C; c-erbB2 (1:200, A 485, DAKO, Glostrup, Denmark) for 30 minutes at room temperature and nm23 (Anti-nm23/NDP kinase A, 1:50, Boehringer Mannheim, Mannheim, Germany) for 2 hours at room temperature. For p53, monoclonal mouse antibody to human p53 protein was used (1:50, DO-7, DAKO, Glostrup) for 2 hours at room temperature.

The avidin-biotin-peroxidase complex technique was used for the localization of proteins. The sections were intensely washed with TBS. Sections were allowed to react with secondary antibody (supersensitive multilink biotinylated IgG, Biogenex, San Ramon, CA) followed by tertiary antibody (supersensitive peroxidase conjugated streptavidin, Biogenex) for 40 minutes each at room temperature. The specific immune reaction was revealed using 3′,3-diaminobenzidine tetrahydrochloride (Sigma, St. Louis, MO) as chromogen and 0.1% H_2O_2 as substrate in 0.05M Tris-HCl (pH 7.6). The sections were counterstained with hematoxylin. Positive controls included breast carcinoma known to exhibit high levels of protein. Negative controls were obtained by omission of the primary antibody.

For all the immunostaining assays, tumors were scored by assessing the site of staining (membrane-c-erbB2, cytoplasm-PRL and nm23, nuclear-p53), and the proportion of stained cells was scored by a semiquantitative score from 0 (negative) to +++ (intense and generalized positivity).

All pathological features were evaluated separately by

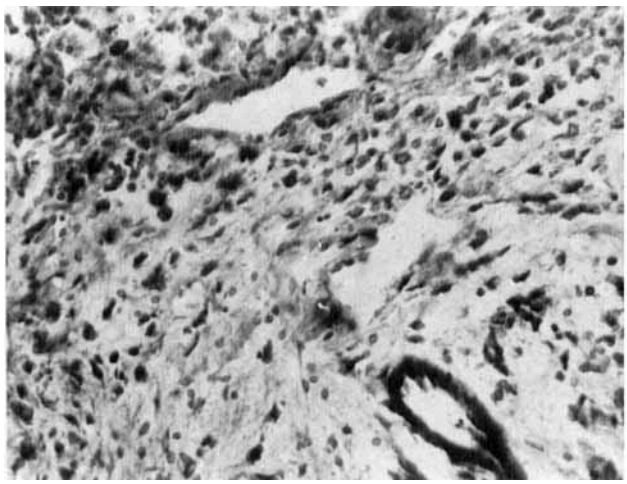

Fig. 2. Immunoperoxidase staining of prolactin in a poorly differentiated breast tumor using polyclonal antibody A 569. Cytoplasmic staining was observed in tumor cell cytoplasm. ×252.

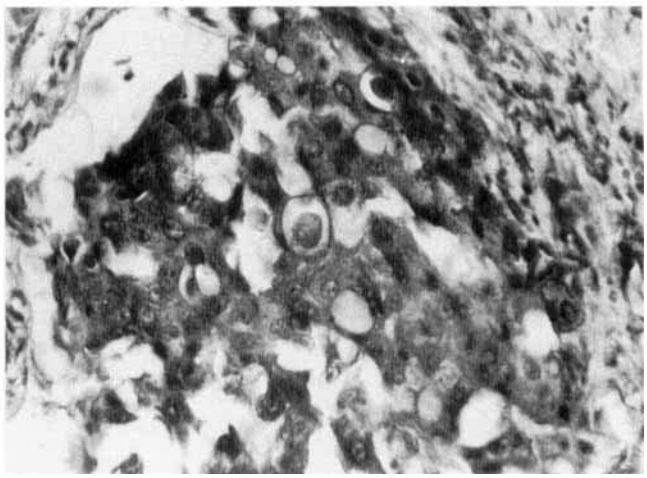

Fig. 3. Moderately differentiated infiltrating duct carcinoma positive for nm23 using polyclonal antibody anti-nm23/NDP kinase. The signal is located in the cytoplasm. ×252.

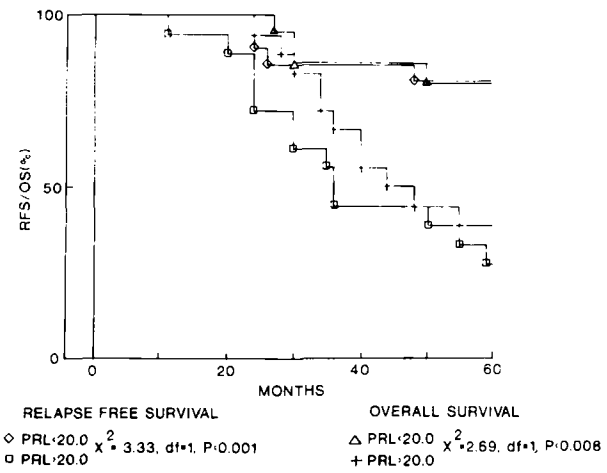

Fig. 4. In a univariate cutpoint analysis of node negative breast cancer patients, those with prolactin (PRL) > 20.0 ng/ml plasma showed significantly reduced relapse-free survival (RFS) and overall survival (OS) compared with prolactin < 20.0 ng/ml plasma.

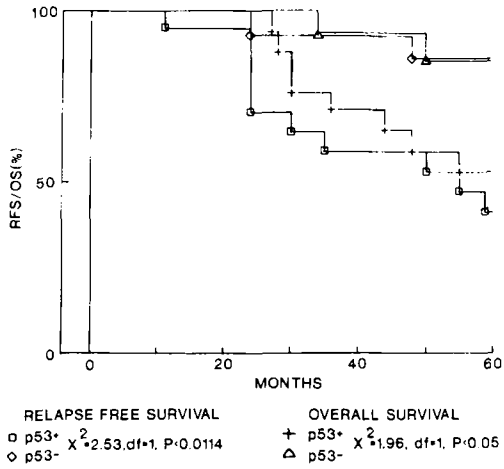

Fig. 5. Significantly reduced five year relapse-free survival (RFS) and overall survival (OS) in node negative breast cancer patients with p53 positive tumors compared to p53 negative tumors.

two investigators who were unaware of the clinical outcome of the patients.

Statistical Analysis

The data were analyzed statistically for significance [13]. The Kaplan and Meier [14] life table analysis was used for RFS and OS, and the significance was calculated according to Chi-square analysis [15].

RESULTS

Clinical Outcome of Patients

RFS was 57% (22/39), whereas OS was 61.5% (24/39) for patients with node negative breast cancer (Fig. 1).

Table II depicts the distribution of markers according to the disease status: (1) patients who developed progressive disease within 5 years, and (2) patients with no evidence of disease (NED) at the end of 5 years. In the subset of patients with progressive disease, the incidence of hyperprolactinemia, PRL and p53 positivity, and nm23 negativity was higher when compared with patients with NED.

IHC Localization

Positive staining for PRL and nm23 was observed in the tumor cell cytoplasm (Figs. 2, 3). Twenty-one out of 31 tumors were positive for PRL immunostaining {+ (N = 14), ++ (N = 7)} and of 26 tumors, 16 showed positive immunostaining for nm23 {+ (N = 5), ++

TABLE III. Relationship of Prognostic Indicators According to Disease Status in Patients With Node Negative Breast Carcinoma

	N	Patients who developed recurrence or metastasis within 5 years	Patients who died within 5 years
Prolactin			
(ng/ml plasma) (N = 39)			
<20.0	21	4*	4**
>20.0	18	13*	11**
Immunohistochemical localization			
Prolactin (N = 31)			
negative	10	2	2
positive	21	7	6
p53 (N = 31)			
negative	14	2***	1****
positive	17	10***	8****
nm23 (N = 26)			
positive	16	3	2
negative	10	5	4
c-erbB2 (N = 26)			
negative	16	7	7
positive	10	3	2
Cathepsin D[a] (N = 31)			
<25.62	17	10	8
>25.62	14	6	6
Estrogen receptors[b] (N = 39)			
positive	18	6	5
negative	21	11	10
Progesterone receptors[b] (N = 39)			
positive	25	9	9
negative	14	8	6

[a] pmol/mg protein.
[b] >10.0 fmol/mg cytosol protein.
N = number of patients.
* <0.00019.
** <0.019.
*** <0.0114.
**** <0.05.

(N = 6), +++ (N = 5)}. Accumulation of p53 protein resulted in IHC localization signal in the nuclei of malignant breast epithelial cells. Of the 31 tumors, 17 showed positive immunostaining {+ (N = 10), ++ (N = 6), +++ (N = 1)}, whereas 14 showed negative immunostaining {−}. We observed membrane and cytoplasmic staining of c-erbB2; however, only membranous staining was considered positive for the purpose of this study. Of the 26 tumors, 10 had membrane positivity {+ (N = 4), ++ (N = 6)} with at least 50% of tumor cells labelled.

Prognostic Value of Plasma PRL

Patients with hyperprolactinemia (18/39) had a significantly shorter RFS and unfavorable prognosis compared to patients with PRL < 20.0 ng/ml plasma (Fig. 4).

Prognostic Value of p53

In a univariate cutpoint analysis, patients with positive staining had a reduced RFS and OS at 5 years than patients with negative p53 staining (Fig. 5). This difference was statistically significant.

Prognostic Value of IHC-PRL, nm23, and c-erbB2, CD, and ER and PR

Statistical differences in RFS and OS were not observed between the two subgroups of each parameter (Table III). Bivariate analysis showed that patients with hyperprolactinemia and p53 overexpression had worse RFS and OS followed by PRL < 20.0 ng/ml and p53 positive group, and hyperprolactinemia and p53 negative group. The patients with PRL < 20.0 ng/ml and p53 negative staining subset had the best prognosis. The difference in RFS and OS was statistically significant between the group of patients with hyperprolactinemia and p53 positive staining, and patients with PRL < 20.0 ng/ml and p53 negative staining (Fig. 6).

DISCUSSION

Our original intention was to extend previous studies on PRL, which showed hyperprolactinemia as an independent predictor of short-term prognosis [10,16], and that a rise in PRL preceded progressive disease in patients

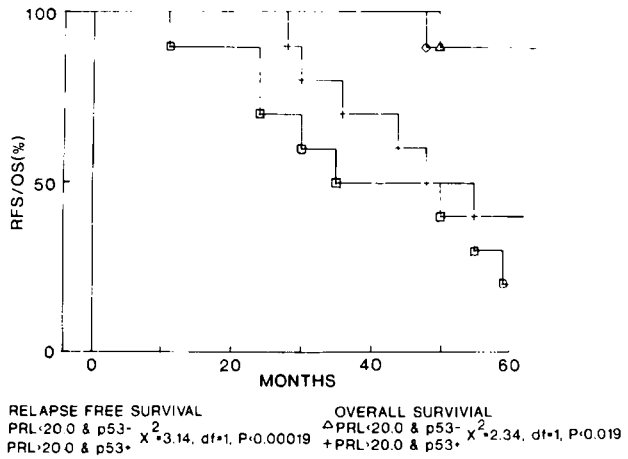

Fig. 6. Five-year relapse-free survival (RFS) and overall survival (OS) in node negative breast cancer patients stratified by plasma prolactin (PRL) and p53 expression. Patients with prolactin >20.0 ng/ml and p53 positive immunostaining had a trend towards faster relapse rate and poor overall survival when compared with prolactin <20.0 ng/ml and p53 negative staining.

with advanced breast cancer [2]. In patients with node negative breast cancer, the present study indicated a strong direct association between hyperprolactinemia and faster recurrence rate and poor outcome. This issue, to differentiate patients with high risk for the development of recurrence or metastases, has become particularly urgent based on the recently demonstrated ability of adjuvant therapy to significantly improve disease-free survival in node negative patients. The results of hyperprolactinemia would thus indicate that aggressive breast cancer may be associated with the production of PRL. We therefore decided to use the IHC approach to study PRL and its significance in node negative breast cancer. Our results indeed showed ectopic production of PRL by breast tumors. Moreover, it was surprising to note that all patients (14/14) had PRL positive staining in the hyperprolactinemic group. We hypothesize that ectopically produced PRL might be acting as one of the major local peptide growth promoters via autocrine or paracrine mechanisms. Furthermore, hyperprolactinemia is thought to contain mitogenic potential that probably facilitates the metastatic process.

Patients with PRL < 20.0 ng/ml plasma exhibited a very favorable RFS and OS. Thus preoperative PRL levels clearly differentiated patients with high risk and low risk for development of recurrence or metastatic disease. In view of the evidence produced by Ward [17] and the current study, there appears some justification in using antiprolactin measures in a restricted group of node negative breast cancer patients who are hyperprolactinemic either at presentation or subsequently.

In the present study, overexpression of p53 protein was also the strongest independent predictor of early recurrence. A mutation results in a prolonged protein half-life and accumulation of the protein in the nucleus [18]. IHC detects this abnormal accumulation [19]. The levels of p53 protein normally increase during late G1 and S-phase. Also, p53 alterations are more often found in advanced breast cancer (Bhatavdekar et al., 1995 unpub. data). In our set of patients, p53 expression was detectable in 55% patients, which was higher when compared with the results of Cattoretti et al. [20] and Ostrowski et al. [21], who found it in 36–46% of node negative breast cancer. The high percent positivity in the present study could be due to the fact that 92% patients had grade II or III tumors. This suggests the possibility that p53 alterations occur more often as a late event in the transformation process, or are associated with an increased metastatic potential. Allred et al. [5] showed strong direct correlation between p53 expression and tumor proliferation rate; both were independently associated with poor prognosis in axillary node negative breast cancer.

The relationship between nm23 and tumor metastatic potential in various human tumor types remains controversial. In human breast cancer, Bevilacqua et al. [22] and Sawan et al. [23] have shown that nm23 expression correlates with tumor metastatic potential; tumors with evidence of metastasis to lymph nodes contained low nm23 expression compared with node negative tumors. In this study, 61.5% of patients with node negative breast cancer exhibited overexpression of nm23 protein. This overexpression did not confer any prognostic significance. However, it still remains to be confirmed in a larger patient population.

Overall c-erbB2 gave no significant additional information when PRL and p53 were considered. A possible explanation for the loss of c-erbB2 predictive ability in this regression model could be that this variable was weakly associated with both PRL and p53. Gasparini et al. [24] suggested that overexpression of c-erbB2 oncoprotein appears to be an important indicator of RFS in stage I–II breast cancer when singly evaluated.

Another prognostic factor that is gaining acceptance is the level of the lysosomal protease cathepsin D [25]. Of the seven studies that specially examined the prognostic significance of CD in node negative patients, two were positive [26,27] and five were negative [28–32]. Our results did not find CD to be of value in predicting the clinical outcome of node negative breast cancer patients.

ER and PR levels predict the likelihood of benefiting from adjuvant tamoxifen therapy [25]. Therefore, it is a valuable marker in the treatment selection of patients with node negative disease. We did not find a significant difference in RFS and OS with ER + PR+ and ER − PR− subsets. When the data were evaluated for menopausal status, neither ER nor PR was found to discriminate for prognosis.

Bivariate analysis showed that when the patients were classified into four subsets on the basis of the cutoff value of plasma PRL and p53 expression, the hyperprolactinemic and p53 positive group of patients had the poorest

prognosis, whereas PRL < 20.0 ng/ml and p53 negative tumors had the best outcome.

From this multiparametric study, we conclude that although the patients had node negative disease, hyperprolactinemia and/or overexpression of p53 protein clearly differentiates patients with high risk for the development of recurrence or metastatic disease. Currently, we are in the process of investigating the expression of PRL mRNA in these patients. We recommend the use of plasma PRL and p53 immunostaining to help identify low risk and high risk groups, so that treatment efforts can be focused on those most likely to benefit. Estimation of plasma PRL and IHC staining of p53 is relatively inexpensive and easy to perform on a routine basis.

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